OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE BY
CLOSTRIDIUM PERFRINGENS

I. Relation of Peroxide to the Over-all Reaction

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Many workers have observed that various species of clostridia can use molecular oxygen as a hydrogen acceptor (Aubel and Houget, 1939; Bard, 1949; Stadtman and Barker, 1949). These reactions are of interest in view of recent growth studies (Holman, 1955), which support the early theory (McLeod and Gordon, 1923b) that growth inhibitory concentrations of peroxide are formed when catalase-negative anaerobes respire in air. It seems, therefore, that characterization of the electron transport reactions of obligate anaerobes, especially with regard to the extent and rate of peroxide formation, might yield information pertinent to the problem of anaerobiosis.

The old yellow enzyme of yeast, a TPNH oxidase (Warburg and Christian, 1933) has generally been taken as a model for the respiratory systems of those flavin-containing organisms that are devoid of hematin catalysts (lactic acid bacteria, clostridia). Thus \( \text{H}_2\text{O}_2 \), formed by two-electron reduction of molecular oxygen, is considered as an obligatory product of flavoprotein respiration. This mechanism is the basis for calling flavoprotein respiration "unphysiological" even for facultative anaerobes. The generalization is not entirely valid, as shown by previous work (Dolin, 1955, 1957b) with Streptococcus faecalis. In this facultative anaerobe, DPNH oxidation takes place as follows:

\[
\text{DPNH} + \text{H}^+ + \text{O}_2 \rightarrow \text{DPN}^+ + \text{H}_2\text{O}_2
\]  

(1)

\[
\text{DPNH} + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{DPN}^+ + 2\text{H}_2\text{O}
\]  

(2)

\[
2\text{DPNH} + 2\text{H}^+ + \text{O}_2 \rightarrow 2\text{DPN}^+ + 2\text{H}_2\text{O}
\]  

(3)

Reactions 1 and 2 are catalyzed by different flavoproteins (DPNH oxidase plus DPNH peroxidase) to give the over-all reaction 3, i.e., flavoprotein respiration without peroxide accumulation.

Flavoprotein enzymes are assumed to account for the peroxide formed by anaerobes; however, few studies dealing with cell-free oxidases for reduced pyridine nucleotides have been carried out. Weber and Kaplan (1954) report that extracts of Clostridium kluveri contain a DPNH oxidase similar in its properties to the old yellow enzyme of yeast (Kaplan, 1955). Mallin and Seeley (1958) have studied DPNH oxidation catalyzed by extracts of Clostridium perfringens, and suggest that a weak DPNH peroxidase activity accounts for the low peroxide levels produced by this organism. The DPNH oxidase of C. perfringens (Dolin, 1957a, 1958), however, resembles neither the old yellow enzyme of yeast nor the flavoprotein oxidase system of S. faecalis. In the anaerobe, reaction 3, the four-electron reduction of oxygen by DPNH, seems to be catalyzed by a single flavoprotein, in a reaction that does not utilize free peroxide as an intermediate. In crude extracts of C. perfringens, the DPNH oxidase is as active (on a specific activity basis) as the cytochrome-containing oxidase in crude extracts of the strict aerobe, Azotobacter agile (A. vinelandii). Soluble four-electron oxidases have been described for catechol and ascorbic acid; however, the four-electron oxidation of DPNH has been considered typical of particulate systems in which cytochrome oxidase is the terminal, oxygen-activating enzyme (Mason, 1957). The finding that C. perfringens contains a soluble enzyme that catalyzes an over-all DPNH oxidation typical of aerobic cells prompted a more detailed investigation into the characteristics and possible physiological significance of this reaction. The papers of this series deal with several properties of the DPNH oxidase.
and related enzymes. Some of the characteristics of these reactions may help to explain the great variability encountered in studies on the occurrence of peroxide in cultures or suspensions of anaerobic bacteria. The first paper describes the properties of the reaction as catalyzed by the crude extract of \textit{C. perfringens}, in order that a comparison may be made to the situation in the intact cell. The second paper deals with the partial purification of the oxidase and with the relation of the oxidase to cytochrome c reductase.

**MATERIALS AND METHODS**

\textit{Enzyme preparations.} \textit{C. perfringens} strain BP6K was kindly supplied by Dr. R. Bard. Twenty-liter cultures of the organism were grown in medium A, according to the procedure of Bard and Gunsalus (1950). Vacuum dried cell preparations and sonic extracts were prepared as previously described for \textit{S. faecalis} (Dolin, 1955), except that sonic treatment was carried out for 8 min. The extracts (supernatant solutions after centrifugation of disrupted suspensions at 25,000 $\times$ G for 30 min) contained 18 to 20 mg of protein per ml and the DPNH oxidase assay varied between 2000 and 2500 units per ml.

\textit{Standard assay for DPNH oxidase.} DPNH disappearance was followed spectrophotometrically at 340 $\mu$m, with a Beckman model DU spectrophotometer (3-ml cuvettes; 1-cm light path). The standard protocol is as follows: potassium phosphate buffer, pH 7.0, 200 $\mu$moles; DPNH, 0.30 $\mu$ mole; enzyme, 2 to 8 units; water to a final volume of 3 ml; temperature, 23 C. The reaction is started by the addition of enzyme. An enzyme unit is defined as that amount of enzyme that causes an optical density change of 0.01 optical density units per min. The initial rate of oxidation (0 to 1 or 0 to 2 min) is proportional to enzyme concentration.

Manometric determinations of oxygen uptake were performed by the conventional Warburg techniques.

\textit{Chemical determinations.} (1) Hydrogen peroxide:—In the range of 0.01 to 0.2 $\mu$ mole of H$_2$O$_2$, the method of Patrick and Wagner (1949) as modified (Dolin, 1957b) was employed (K-molybdate method). For the range 2 $\times$ 10$^{-4}$ to 3 $\times$ 10$^{-2}$ $\mu$ moles, a modification of the luminol method of Schales (1939) was used.

In order to reduce the peroxide blank, catalase is added to the hemin-luminol reagent (3 mg of crystalline catalase per 40 ml of reagent). After standing for 15 to 30 min, the reagent is ready for use. The chemiluminescence accompanying the oxidation of luminol by peroxide is determined with a liquid nitrogen cooled photomultiplier connected to a quantum counter (Strehler, 1951). In a typical experiment, 0.001 $\mu$ mole of H$_2$O$_2$ causes the counting rate to increase 17-fold over the blank. Since the sensitivity of the reagent doubles in 4 hr, standard curves are run at hourly intervals. To correct for quenching effects, standard curves are run in the presence of appropriate control additions and experimental determinations are checked with internal standards.

(2) Other determinations:—Protein was determined by the method of Lowry et al. (1951) and DPNH by use of the molecular extinction coefficient, $6.22 \times 10^4$ cm$^{-2}$ per mole at 340 $\mu$m (Horecker and Kornberg, 1948).

Enzymatically reduced DPN (Na salt) was obtained from Sigma, luminol (3-aminophthal-hydrazide) from Aldrich Chemical Co., and hemin from Nutritional Biochemicals Corp. Vacuum-dried cells of \textit{C. kluyveri}, and crystalline catalase were purchased from Worthington Biochemical Corporation.

**EXPERIMENTAL RESULTS**

\textit{Balance for DPNH oxidation.} Cell-free extracts of \textit{C. perfringens} catalyze a rapid, cyanide-insensitive oxidation of DPNH to DPN (equation 3). The specific activity (0.5 to 0.65 $\mu$ mole of DPNH oxidized per mg of protein per min at 23 C) is as great as that found for crude extracts of the highly aerobic organism, \textit{A. agile} (0.8 $\mu$ mole per mg of protein per min at 35 C) (Brümmel et al., 1957). Documentation of the over-all reaction is shown in table 1. Within experimental error, 0.5 mole of oxygen is utilized per mole of DPNH oxidized and no peroxide is found. Furthermore, hydrogen peroxide is not decomposed by the complete system or its individual components. Quantitative recoveries of added peroxide, in the range 0.2 to 0.003 $\mu$ mole per 3 ml are obtained, even after incubation for 20 min. Although stoichiometric amounts of peroxide are not formed, traces ($2 \times 10^{-7}$ to $3 \times 10^{-4}$ $\mu$mole) may be detected with the luminol technique. This point will be amplified later. DPN is the product of the reaction, as shown by the quantitative recovery of DPNH upon reduction.
TABLE 1

<table>
<thead>
<tr>
<th>Additions</th>
<th>DPNH Oxidized</th>
<th>O₂ Uptake</th>
<th>H₂O₂ Formed</th>
<th>O₂/DPNH</th>
<th>H₂O₂/DPNH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. C. perfringens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>0.77</td>
<td>0.38</td>
<td>0</td>
<td>0.49</td>
<td>—</td>
</tr>
<tr>
<td>Expt 2</td>
<td>0.99</td>
<td>0.45</td>
<td>0</td>
<td>0.46</td>
<td>—</td>
</tr>
<tr>
<td>2. C. perfringens + menadione</td>
<td>0.30</td>
<td>—</td>
<td>0.20</td>
<td>—</td>
<td>0.67</td>
</tr>
<tr>
<td>3. C. kluwyeri</td>
<td>0.16</td>
<td>—</td>
<td>0.17</td>
<td>—</td>
<td>1.06</td>
</tr>
</tbody>
</table>

1. Double side arm Warburg vessels, total volume 6 ml. First side arm: DPNH, 2.4 μmoles in 0.1 ml of H₂O. Second side arm: 10 n KOH, 0.02 ml. Cup: potassium phosphate buffer, 50 μmoles, pH 7.0; sonic extract, 20 mg of protein per ml, 0.1 ml for experiment 1, 0.2 ml for experiment 2; H₂O to 1 ml final volume. Temperature, 23 C. Reaction run for 12 min, and then alkali tipped in.

2. Standard spectrophotometric assay, except for menadione, 0.3 μmole; sonic extract, 0.2 mg of protein. Reaction run for 10 min, and stopped with 0.05 ml of 10 n H₂SO₄.

3. Standard assay, 9 min. Sonic extract (dialyzed with stirring against 500 volumes 0.02 m potassium phosphate buffer, pH 7.0, 4 C, 4 hr), 0.6 mg of protein.

of the oxidized product with alcohol and alcohol dehydrogenase (Racker, 1950) at pH 9.7. The oxidation of DPNH by C. perfringens did not go to completion, under the conditions of table 1, because of an inhibitory reaction caused by high concentrations of substrate. This effect will be described in a later section. The balance of equation 3 is obtained with enzyme at all stages of purity, including the purest fractions prepared by zone electrophoresis (Dolin, 1959). The experiments summarized in numbers 2 and 3, table 1, serve as positive controls for peroxide formation. In the presence of 10⁻⁴ m menadione, the oxidation of DPNH by C. perfringens extracts is stimulated about 3-fold, and two-thirds of the theoretical yield of peroxide is formed.

This finding would be explained if two separate enzymes were involved, the DPNH oxidase and a DPNH-menadione reductase that would yield peroxide via the autoxidation of reduced menadione. Later work showed that the extracts do, in fact, contain a menadione reductase that can be completely separated from the oxidase.

In confirmation of the report of Weber and Kaplan (Kaplan, 1955) that the DPNH oxidase of C. kluwyeri resembles the old yellow enzyme of yeast, number 3 shows that DPNH oxidation, catalyzed by a dialyzed sonic extract of this organism, results in the formation of stoichiometric yields of peroxide. It is apparent, therefore, that the oxidase of C. perfringens is different from the "typical" flavoprotein oxidase.

Kinetics. The time course for DPNH oxidation is shown in figure 1. Over a period of 14 min, the initial oxidation rate decreases about 6-fold. This loss of activity is not caused by depletion of either DPNH or oxygen. If more DPNH is added at 14 min, the rate is not affected; however, on the addition of fresh enzyme, the initial rate is again obtained. Similarly, it can be shown that...
The table constant for inactivation of DPNH oxidation, Clostridium perfringens.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Initial Oxidation Rate</th>
<th>$k_i$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C. \text{perfringens}$ extract</td>
<td>5.4</td>
<td>0.14</td>
<td>5.0</td>
</tr>
<tr>
<td>+ catalase, 1 mg</td>
<td>5.3</td>
<td>0.049</td>
<td>14.0</td>
</tr>
<tr>
<td>+ catalase, 0.2 mg</td>
<td>5.5</td>
<td>0.049</td>
<td>14.0</td>
</tr>
<tr>
<td>+ catalase, 0.02 mg</td>
<td>5.5</td>
<td>0.065</td>
<td>11.0</td>
</tr>
<tr>
<td>+ catalase, 0.002 mg</td>
<td>5.1</td>
<td>0.10</td>
<td>6.7</td>
</tr>
<tr>
<td>+ catalase (heated), 1 mg</td>
<td>5.3</td>
<td>0.13</td>
<td>5.3</td>
</tr>
<tr>
<td>+ cytochrome c, 0.05 mg</td>
<td>5.0</td>
<td>0.14</td>
<td>5.0</td>
</tr>
<tr>
<td>+ $H_2O_2$, $3 \times 10^{-4}$ M</td>
<td>4.1</td>
<td>0.31</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Standard assay, except for catalase and cytochrome c as shown. Sonic extract $C. \text{perfringens}$ (dialyzed against 500 volumes of potassium phosphate buffer, pH 7.0, 5 hr, 4°C) 0.1 mg of protein, $H_2O_2$, where present, incubated with enzyme for 5 min and reaction started by addition of DPNH. $k_i =$ first-order rate constant for enzyme inactivation; $\alpha =$ half-time for inactivation.

Table 2: Effect of catalase on inactivation rate of DPNH oxidase, Clostridium perfringens.

Figure 2. First-order decay of DPNH oxidase activity, Clostridium perfringens extract. Standard assay, except 0.6 μ mole of DPNH used. Sonicate extract, (dialyzed against 500 volumes of 0.02 M potassium phosphate, pH 7.0, 5 hr with stirring), 6.5 units. ●, without catalase. ○, with 0.2 mg crystalline catalase. The change in absorbance at 340 mμ over a 1-min interval is taken as the rate at the beginning of that interval.

Oxygen is not the limiting factor and in fact the time course of the oxidation is identical in oxygen saturated solution. The loss in activity does not take place in the absence of substrate. Neither DPN nor the primary acid modification product of DPNH (Rafter et al., 1954) is inhibitory. DPNH purified by paper-strip electrophoresis produces the same inhibition as untreated DPNH. These findings, together with the fact that the time course is not affected in the presence of 0.1 per cent versene, seem to rule out heavy metal contamination, or DPN formation as the inhibitory factor.

The apparent kinetics of DPNH disappearance depend upon the initial ratio of DPNH to enzyme and can appear to be either first or second order. These results are, however, attributable to the fact that enzyme activity is decaying with first order kinetics, as shown in figure 2. Under conditions of substrate saturation, enzyme activity disappears in a first-order reaction, with a rate constant of 0.13 min$^{-1}$. A large portion of this inactivation can be attributed to the traces of peroxide formed during oxidation (figure 2, table 2). In the presence of catalase, the rate constant for inactivation, $k_i$, is reduced about 2.9-fold. The effect of catalase is discernible at a level of 2 μg per 3 ml and the maximal effect is obtained somewhere between 20 and 200 μg per 3 ml. Cytochrome c or heated catalase are without effect. The initial oxidation rates are not affected by catalase. Hydrogen peroxide, in a final concentration of $3 \times 10^{-4}$ M increases the inactivation rate about 2-fold compared to the complete system in the absence of catalase, or 6-fold compared to the rate obtained in the presence of excess catalase.

DPNH may be inhibitory, however, since (a) catalase does not completely prevent the inhibition, (b) under anaerobic conditions, in which no DPNH oxidation or peroxide formation can be detected, the inactivation still takes place at a rapid rate (the kinetics of the anaerobic process have not been investigated), and (c) peroxide, by itself, does not cause first-order decay of enzyme activity. In the presence of $3 \times 10^{-4}$ M peroxide, an immediate inhibition of 25 to 30 per cent is produced, but no further inhibition of the initial oxidation rate is obtained even after incubation for 20 min of peroxide and enzyme. However, DPNH oxidation for 14 min results in an 85 per cent inhibition of enzyme activity. In this oxidation, peroxide concentrations of the order of 2 to $3 \times 10^{-4}$ M may be formed.

Thus the inhibition phenomenon is complex, and requires the presence of both DPNH and peroxide; it seems to be different from the “reac-
tion inactivation" described for other four-electron oxidases, such as ascorbic acid oxidase (Powers and Dawson, 1943). "Reaction inactivation" requires substrate turnover for inhibition to occur. A DPNH-dependent decay of activity is also found with the flavoprotein, DPNH peroxidase (Dolin, 1957a). In this system, however, the first-order decay is prevented by the physiological oxidant, peroxide.

**Inhibition caused by high DPNH concentrations.** Superimposed upon the first-order decay, there occurs, at high DPNH concentrations, an immediate inhibition as shown in figure 3. The optimal rate of oxidation requires about 0.3 μmole of DPNH per ml. At higher concentrations, the initial oxidation rate decreases almost as a direct linear function of the DPNH concentration. Catalase does not prevent this inhibition. Since the first-order decay constant does not vary appreciably with DPNH concentration over the range 0.1 to 0.7 μmole per ml, the strong inhibitions detected at DPNH concentrations in excess of 0.3 μmole per ml are different from those caused by the first-order decay process.

The immediate inhibitions may reflect the binding of an additional molecule of DPNH to the enzyme. Neither DPN nor DPNH that has been oxidized to DPN enzymatically (DPNH peroxidase) is inhibitory in high concentration.

*Figure 3. DPNH oxidase* *Clostridium perfringens.* Enzyme activity versus DPNH concentration. Standard assay, except for DPNH concentrations as shown. Enzyme as in figure 2; 200 μmoles of potassium phosphate buffer. Rates plotted are those for the 0 to 1 min interval after the addition of enzyme.

*Figure 4. DPNH oxidation by sonic extract of* *Clostridium perfringens.* H₂O₂ formation vs. flavinadenine dinucleotide (FAD) concentration. Standard assay, except for FAD as shown. Sonic extract (dialyzed under conditions given in figure 2, 10 hr), 4 units. Reaction run for 20 min, stopped with 0.05 ml of 10 N H₂SO₄, and reaction mixtures centrifuged at 4 C. H₂O₂ was determined by the KI-molybdate method.

**Sources of H₂O₂ during DPNH oxidation.** (1) Enzymes using autoxidizable hydrogen acceptors:—It has been mentioned that high concentrations of peroxide can be formed through the action of a menadione reductase. Crude extracts also contain an enzyme or enzymes that yield peroxide when DPNH oxidation is carried out in the presence of flavinadenine dinucleotide (FAD). Figure 4 shows the variation in peroxide yield as a function of FAD concentration. This peroxide-forming reaction is not accompanied by an increased rate of DPNH oxidation. Peroxide production probably results from enzymatic reduction and spontaneous reoxidation of free FAD. Although substrate concentrations of free flavins (FAD, FMN, and riboflavin) can be reduced by DPNH when high concentrations of crude extract are used, neither FMN nor riboflavin cause peroxide formation when normal concentrations of enzyme are employed. This suggests that under normal assay conditions, FMN and riboflavin are not reduced rapidly enough to support peroxide formation.

The purified DPNH oxidase (Dolin, 1959) does not carry out the FAD-dependent, peroxide-forming reaction nor does it catalyze the reduction of free flavins. Actually, the purified oxidase
(2) DPNH oxidase—In the absence of autodizable acceptors, no H₂O₂ is detected by the KI-molybdate method. With the luminol reaction (Methods section), it can, however, be demonstrated that, during DPNH oxidation, peroxide levels in the range 2 × 10⁻⁷ to 3 × 10⁻⁶ M are formed. Peroxide formation requires the presence of DPNH and native (unheated) enzyme. The peroxide produced in this reaction is immediately decomposed by catalase, and is therefore probably hydrogen peroxide.

With crude extracts, the yield of H₂O₂ (moles of H₂O₂ formed per mole of DPNH oxidized) in the initial stages of the oxidation is 5 to 6 per cent. Under the same conditions, electrophoretically purified enzyme (Dolin, 1959) gives yields of approximately 1 per cent. This difference is probably attributable to the presence, in crude extracts, of contaminating autoxidizable flavoproteins. As would be expected from the dependence of the inactivation rate on peroxide concentration, the purified enzyme is inactivated more slowly (kᵢ = 0.08 min⁻¹) than the crude enzyme. With both types of preparation in a 10- to 12-min experiment, about one-half of the final concentration of peroxide is obtained in the first 1 to 1.5 min. The slow increase in peroxide concentration after 1.5 min affects the rate of enzyme inactivation. Examination of figure 2 shows that although a straight line can be fitted to all the points of the first-order plot, there is a difference between the initial and final slopes. The values for kᵢ reported in this paper are averages obtained in a 12- to 14-min experiment. With many different batches of sonic extract, these values have been rather constant (0.12 to 0.15 min⁻¹).

Role of H₂O₂ in the over-all oxidation. The question arises whether the peroxide concentrations found represent steady-state levels of an active intermediate or whether the peroxide functions merely as a toxic agent. The difference in behavior between crude and purified enzyme supports the latter hypothesis. Furthermore, if peroxide concentrations in the range demonstrated to occur are intermediates, it should be possible to show that such concentrations are utilized anaerobically in the presence of enzyme and substrate. Such an experiment is summarized in figure 5. With an amount of extract of C. perfringens capable of oxidizing 0.054 μmole of DPNH in 1 min, neither 0.003 nor 0.009 μmole
of peroxide was decomposed in the presence or absence of DPNH. At the end of the experiment, the aerobic activity of the enzyme was checked, and it was found that there was still enough activity left to account for the decomposition of the peroxide within 0.25 to 1 min, if the over-all oxidation is the sum of equations 1 and 2. Aerobically, DPNH oxidation in the presence of added $H_2O_2$ leads to the formation of additional peroxide, the yield being about the same as that found in the absence of initially added peroxide.

In contrast to these findings, it can be seen that a crude extract of S. faecalis, which oxidizes DPNH via reactions 1 and 2, utilizes 0.009 μmole of peroxide to completion in an enzyme and substrate-dependent reaction. On an activity basis, the extract of S. faecalis was present in 0.37 times the concentration of the enzyme from C. perfringens. It appears, therefore, that even coenzyme level concentrations of free hydrogen peroxide are not utilized by the DPNH oxidase of C. perfringens.

Specificity for reductant and oxidant. Neither TPNH nor reduced cytochrome c (horse heart) serve as substrates for the oxidase. The menadione and flavin reactions have been discussed.

Neither $H_2O_2$ as discussed, nor nitrate serve as oxidant for the DPNH oxidase of C. perfringens. Ascorbic acid, which (presumably via a one-electron intermediate) is required by certain DPNH oxidases of peas, yeast, and bacteria (Nason et al., 1954; Kern and Racker, 1954; Vernon and White, 1957) is inhibitory to the oxidase of C. perfringens (doubles the decay rate). The relation between cytochrome c reductase and DPNH oxidase in C. perfringens will be described in the second paper of this series.

Inhibitors. The peroxide inhibition described may indicate that a sensitive —SH group is necessary for the oxidation. Table 3 shows that the enzyme is also very sensitive to p-chloromercuribenzoate. The lack of inhibition by cyanide and 8-hydroxyquinoline suggests that the oxidase is not a metallo-enzyme of the hematin or Cu oxidase type; however, the azide effect apparently does not fit this interpretation. There are indications, on the other hand, that azide may function at other than metallic sites (Henry and Henry, 1946) and may, in fact, be an inhibitor of quinone-dependent reactions (Chase, 1942).

Presumptive evidence for a flavin prosthetic group is offered by the atabrine inhibition (Haas, 1944), which can be partially prevented by FAD. More direct evidence for the flavoprotein nature of the enzyme will be presented in the second study.

**DISCUSSION**

Electron transport to oxygen is potentially useful to a fermentative organism, whether or not the transport is coupled with phosphorylation. The presence of an accessory path to oxygen

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**TABLE 3**

Inhibitors of DPNH oxidase, Clostridium perfringens

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percentage Inhibition of Initial Oxidation Rate</th>
<th>$k_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>3 $\times$ $10^{-4}$M</td>
<td>25</td>
</tr>
<tr>
<td>1.33 $\times$ $10^{-4}$M</td>
<td>67</td>
<td>$\sim$0.59</td>
</tr>
<tr>
<td>6.6 $\times$ $10^{-5}$M</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>p-Chloromercuribenzoate, 1.7 $\times$ $10^{-6}$M</td>
<td>44</td>
<td>0.09</td>
</tr>
<tr>
<td>1.7 $\times$ $10^{-6}$M</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>1.7 $\times$ $10^{-6}$M</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Na arsenite, 3 $\times$ $10^{-6}$M</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Atabrine, 1 $\times$ $10^{-4}$M</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>3.3 $\times$ $10^{-4}$M</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>6.6 $\times$ $10^{-4}$M</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>6.6 $\times$ $10^{-4}$M + FAD, 1.3 $\times$ $10^{-4}$M</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>1.0 $\times$ $10^{-5}$M</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>KCN, 1 $\times$ $10^{-5}$M</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>Na azide, 10$^{-6}$M</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>8-Hydroxyquinoline, 6.6 $\times$ $10^{-6}$M</td>
<td>10</td>
<td>0.17</td>
</tr>
<tr>
<td>o-Phenantroline, 1.7 $\times$ $10^{-6}$M</td>
<td>11</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Standard assay, except that inhibitors were incubated with enzyme for 5 min, and the reaction was started by the addition of DPNH. Enzyme, sonic extract C. perfringens, 6 to 7 units.
can make available to the organism substrates that might not be oxidizable anaerobically, for lack of hydrogen acceptors. In the aerobic reactions, energy could be conserved through substrate-linked phosphorylations, just as in the fermentative series of reactions. However, for organisms devoid of iron-porphyrin catalysts, the usefulness of oxygen may be limited by two factors: (a) the nature of the flavoprotein respiratory mechanism used, i.e., whether peroxide or water is the product of oxygen reduction, and (b) the sensitivity of key enzymes to peroxide.

Whether oxygen is inhibitory, innocuous, or metabolically useful may depend upon interactions between these factors. For instance, the inability of *C. perfringens* to use oxygen as a physiological electron acceptor may be understandable on the following grounds: (a) the DPNH oxidase, although it catalyzes the reduction of oxygen to water, can be inhibited even by the traces of peroxide formed during the oxidation; (b) other enzymes, which use autoxidizable acceptors (i.e., FAD, menadione) cause the rapid formation of peroxide concentrations that are inhibitory to several dehydrogenases of the organism (Grunberg-Manago *et al.*, 1952) and to growth (McLeod and Gordon, 1923a). When autoxidizable acceptors are absent, or present in low concentration, DPNH oxidation takes place primarily through the four-electron oxidase, and peroxide is formed slowly. A mechanism such as this might account, in part, for the ability of *C. perfringens* to grow under oxygen tensions that inhibit the growth of stricter anaerobes (Gordon *et al.*, 1953), and for the "aerotolerance" shown by various other clostridia (Breed *et al.*, 1957).

Since the peroxide level in *C. perfringens* is controlled mainly by the activity of oxidases that function with autoxidizable acceptors, the concentration of such acceptors becomes an important physiological consideration (cf. figure 4). Similar dependence upon the concentration of exogenous acceptors might account for some of the contradictory reports concerning the ability of various anaerobes to form peroxide in the presence of air. Holman (1955) has reviewed the literature dealing with peroxide formation by anaerobes.

If efficient mechanisms for peroxide decomposition were present, oxygen might be noninhibitory for many clostridia. This idea is supported by the work of Holman (1955) who has shown that various species of anaerobes, including *C. perfringens*, can be grown on semisolid or solid media, under a solution of catalase. A DPNH peroxidase mechanism, such as that described for *S. faecalis* (Dolin, 1955, 1957b), could serve a similar function. The DPNH peroxidase activity reported for fresh sonic extracts of *C. perfringens* (Mallin and Seeley, 1958), however, seems to be much too weak to compete with the peroxide-forming reactions.

By analogy to the mechanisms that have been postulated for four-electron transfer reactions (Mason, 1957), some suggestions can be made concerning the mechanism for DPNH oxidation by *C. perfringens*. The over-all reaction (equation 3) could involve as intermediates E·H₂O₂ or doubly reduced enzyme, E·(H₂)₂ or E·(DPNH)₂. The corresponding mechanisms would be (without writing all the possible intermediate complexes):

$$E + DPNH \rightarrow E \cdot DPNH$$
$$E \cdot DPNH + H^+ + O_2 \rightarrow E \cdot H_2O_2 + DPN^+$$
$$E \cdot H_2O_2 + DPNH + H^+ \rightarrow E + DPN^+ + 2 H_2O$$

*Mechanism 1*

$$E + 2 DPNH \rightarrow E \cdot (DPNH)_2$$
$$E \cdot (DPNH)_2 + 2 H^+ + O_2 \rightarrow E + 2 DPN^+ + 2 H_2O$$

*Mechanism 2*

Other mechanisms, involving E·O₂ and E·O complexes are considered plausible for certain metallo-enzymes. Model reactions may be found in the review of Mason (1957). Results reported for two other flavoproteins may be pertinent. The lactate oxidase of *Mycobacterium phlei* (Sutton, 1957) catalyzes the oxidation of lactate to acetate and CO₂, without stoichiometric peroxide production. It is believed that enzyme-bound pyruvate and peroxide are intermediates. Weber *et al.* (1956) have presented evidence for the occurrence of enzyme-bound peroxide as an intermediate in the reduction of cytochrome c by xanthine oxidase. If a bound form of peroxide is the intermediate in the clostridial system, it is not in equilibrium with free hydrogen peroxide.

**SUMMARY**

A soluble, cyanide-insensitive oxidase from *Clostridium perfringens* catalyzes the reduction of oxygen to water, with reduced diphosphopyridine nucleotide as the hydrogen donor. The activity
of the oxidase in crude extracts is as great as that of the cytochrome-containing reduced diphosphopyridine nucleotide oxidase of the strict aerobe, Azotobacter agile (A. vinelandii). Traces of peroxide (2 × 10⁻⁷ to 3 × 10⁻⁴ M) are formed during the oxidation of reduced diphosphopyri-
dine nucleotide by the clostridial enzyme; how-
ever, direct experiment shows that such coenzyme levels of free peroxide are not intermediates in the over-all oxidation mechanism.

Two types of inhibition reaction caused by re-
duced diphosphopyridine nucleotide may be rec-
ognized. One type is dependent upon the per-
oxide concentration and leads to first order decay of enzyme activity. The second is detected at high concentrations of reduced diphosphopyridine nucleotide and causes immediate inhibitions, which are directly proportional to the concentra-
tion of the reduced nucleotide.

Crude extracts of C. perfringens also contain reductases for flavinadenine dinucleotide and menadione. Autoxidation of the reduced form of the latter compounds leads to high concentra-
tions of peroxide. These reductase activities are not properties of the purified reduced diphos-
phopyridine nucleotide oxidase.

The characteristics of the reduced diphospho-
pyridine nucleotide oxidation reactions, as cata-
lized by crude and purified extracts, are dis-
cussed in relation to the mechanism of anaer-
obiosis.

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